PHYSIOLOGY

The Expression of Pseudogene Cyclin D2 mRNA in the Human Ovary May Be a Novel Marker for Decreased Ovarian Function Associated with the Aging Process¹

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Purpose: Our purpose was to investigate the expression pattern of cyclin D2 and pseudogene cyclin D2 mRNA in the human ovary with age.

Methods: After extraction of the total RNA from ovarian tissues of 23 premenopausal patients, cyclin D2 and pseudogene cyclin D2 mRNAs were measured by the reverse transcription–polymerase chain reaction technique using cyclin D2 and pseudogene cyclin D2 specific primers. Analysis of the cyclin D2 and pseudogene cyclin D2 mRNA expression pattern with age and correlation analysis were carried out.

Results: A 489-bp cyclin D2 band and a 441-bp pseudogene cyclin D2 mRNA band were detected in the human ovarian tissue. While cyclin D2 mRNA expression showed a decreasing tendency with age ($P = 0.17$), pseudogene cyclin D2 mRNA expression increased with age ($P < 0.05$). Pseudogene cyclin D2 mRNA expression showed a negative correlation with cyclin D2 mRNA ($R = -0.35$, $P < 0.03$).

Conclusion: The expression of pseudogene cyclin D2 mRNA in the human ovary increases with age, which may be a novel marker for decreased ovarian function associated with the aging process.

KEY WORDS: Human ovary; cyclin D2; pseudogene cyclin D2.

INTRODUCTION

The D-type cyclins (D1, D2, and D3) have been known as cell cycle activators in the ovary (1). Among these cyclins, cyclin D2 has been reported to have a pivotal role in granulosa cell proliferation in the mouse and rat ovary and is specifically located in the granulosa cells of growing follicles in the rat ovary (2). Also, the critical role of cyclin D2 in hormone-dependent follicular growth is demonstrated in mice null for cyclin D2 (3). However, little is known regarding the pseudogene cyclin D2, which is incidentally identified during reverse transcription–polymerase chain reaction (RT-PCR) using a cyclin D2 primer in the human ovary. In this study, we investigate the expression pattern of cyclin D2 and pseudogene cyclin D2 mRNA in the human ovary with age.

MATERIALS AND METHODS

Patients

Ovarian tissues of 23 premenopausal patients (aged 21 to 52) who underwent laparotomy or pelviccopy for benign gynecologic reasons were obtained after informed consent. Patients were divided into four subgroups according to their age: Group I, 20 to 29 ($n = 6$); Group II, 30 to 39 ($n = 5$); Group III, 40 to 49 ($n = 7$); and Group IV, 50 to 52 ($n = 5$).

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Fig. 1. Cyclin D2 and pseudogene cyclin D2 mRNA expression in the human ovary by RT-PCR.

The specific gynecologic indications for surgery were as follows: Group I, ovarian tumors (one teratoma, four ovarian endometrioses, and one mucinous cystadenoma); Group II, four uterine myomas and one ovarian endometriosis; and Groups III and IV, uterine myomas with or without ovarian cysts. This study was approved by the Ethical Committee of the Samsung Medical Center.

RT-PCR

Total RNA was extracted from ovarian tissues with TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. Total RNA (2 μ g) was reverse-transcribed in a final 25 μ l of Tris buffer (50 mM, pH 8.3) supplemented with KCl (75 mM), MgCl₂ (3 mM), DTT (5 mM), deoxynucleotide triphosphate (0.5 mM each) (Gibco-BRL), oligo (dT) (1.5 M), and Moloney murine leukemia virus (M-MLV) reverse transcriptase (200 U; Promega) at 42°C for 60 min. PCR amplification was performed in a final 50- μ l reaction volume containing 2 μ l of reverse-transcribed cDNA, a 0.4 M concentration of each primer, 1 U of Taq DNA polymerase (Promega, Madison, WI), 1 $\times$ Taq DNA polymerase buffer, MgCl₂ (2.5 mM for cyclin D2 and GAPDH, 1.5 mM for pseudogene cyclin D2), and deoxynucleotide triphosphate (0.2 mM each). The PCR reaction was conducted in a Progene Thermal Cycler with the following program: denaturing for 1 min at 95°C, annealing for 45 sec at 56°C (for cyclin D2), at 64°C (for pseudogene cyclin D2), and at 60°C (for GAPDH), and elongation for 2 min at 74°C for a total of 30 cycles (for cyclin D2), 28 cycles (for GAPDH), and 33 cycles (for pseudogene cyclin D2), except for the last cycle, for which the elongation

step was prolonged to 10 min at 74°C. The amount of template cDNA used in each reaction was normalized to the amount of GAPDH mRNA. Amplification of template cDNA was in the linear range for the number of PCR cycles, and RT-PCR of GAPDH transcript was performed using the same amount of cDNA from each sample as a template. PCR products were visualized by electrophoresis on 1.5% (w/v) agarose gels.

Oligonucleotide Primers

Based on the cDNA sequences available in GenBank, oligonucleotide PCR primers were selected with the assistance of a computer program (DNA STAR) designed to optimize the GC content and melting temperature and to minimize hairpin and dimer formation. PCR primers were as follows: cyclin D2, for 5'-TACTTCAAGTGCCTGCAGAA GGAC-3', with cyclin D2 reverse, rev 5'-TCCC ACACTTCCAGTT GCGATCAT-3', and GAPDH, for 5'-ACCACAGT CCATGCCATCAC-3', with GAPDH reverse, rev 5'-TCCACCACCCTGTTGCT GTA-3'. These resulted in products of 489 and 452 bp, respectively. PCR products were sequenced to verify their identity and homology to corresponding cDNA sequences in GenBank. A 577-bp shifted band was detected by RT-PCR using cyclin D2 primers. Sequencing of the 577-bp band showed 100% homology with pseudogene cyclin D2 mRNA. Based on the sequencing results and GenBank, the following specific primer pairs were designed for pseudogene cyclin D2 for 5'-CGAGGCAGGCAGG GAAGC-3' and reverse 5'-CATCGAAGTGGCACCAATCAAAT-3', which resulted in a product of 441bp.

Fig. 2. Cyclin D2 and pseudogene cyclin D2 mRNA expression in the human ovary. (A) Cyclin D2 and pseudogene cyclin D2 mRNA expression with age (mean $\pm$ SE). (B) Correlation of cyclin D2 and pseudogene cyclin D2 mRNA expression.

Densitometry

Gel images were scanned with the Gel Documentation System (Gel Doc 1000; Bio-Rad, Hercules, CA), and relative densities were analyzed using the Multi-analyst fingerprinting program (version 1.1). The relative densities of the bands are expressed as arbitrary absorbance units per area.

Statistical Analysis

The cyclin D2 mRNA and pseudogene cyclin D2 mRNA expression patterns with age and the correlation between these two parameters were tested by Jonckheere–Terpstra test and Spearman’s correlation analysis, respectively. Statistical significance was considered at a *P* value of less than 0.05.

RESULTS

A 489-bp cyclin D2 gene and a 577-bp shifted band were detected by RT-PCR using cyclin D2 primers (Fig. 1A). The sequencing results showed that the 577-bp shifted band was 100% homologous with pseudogene cyclin D2 mRNA, and the 489-bp PCR product was cyclin D2 mRNA. Based on sequencing results, we designed new specific primers for pseudogene cyclin D2 and performed RT-PCR, which resulted in 441-bp pseudogene cyclin D2 mRNA (Fig. 1B). After normalization with GAPDH mRNA, the intensities of cyclin D2 mRNA according to age groups were 1.09 ± 0.35 , 0.90 ± 0.17 , 0.73 ± 0.17 , and 0.40 ± 0.13 , respectively (arbitrary units; mean $\pm$ SE). Cyclin D2 mRNA expression showed a decreasing tendency with age ($P = 0.17$). The intensities of the pseudogene cyclin D2 mRNA according to age groups were 0.09 ± 0.01 , 0.13 ± 0.03 , 0.34 ± 0.11 , and 0.35 ± 0.20 , respectively. Pseudogene cyclin D2 mRNA expression increased with age ($P < 0.05$) (Fig. 2A). Pseudogene cyclin D2 mRNA expression showed a negative correlation with cyclin D2 mRNA expression ($R = -0.35$, $P < 0.03$) (Fig. 2B).

DISCUSSION

Mammalian D-type cyclins are differentially and redundantly expressed during the G1 phase of the cell cycle. Human cyclin D2 genes share a very high similarity with each other and with their murine counterparts but considerably less similarity with other human cyclins (4). Findings such as remaining small ovarian follicles, failed ovulation, and impairment of granulosa cell proliferation in mice null for cyclin D2 suggest that it is essential for normal ovarian function (3). And cyclin D2 mRNA expression in human luteinized granulosa cells correlated positively with the clinical parameters of ovarian function (our data submitted for publication). So there might be a negative correlation between cyclin D2 mRNA expression and age. In this study, 489-bp cyclin D2 and 441-bp pseudogene cyclin D2 RT-PCR products were detected in human ovarian tissue (Fig. 1). As expected, expression of cyclin D2 mRNA showed a decreasing tendency ($P = 0.17$). But pseudogene cyclin D2 mRNA expression increased with age

($P < 0.05$) and showed a negative correlation with cyclin D2 mRNA expression ($R = -0.35$, $P < 0.03$). Based on these results and the positive correlation of cyclin D2 expression with clinical parameters of ovarian function, pseudogene cyclin D2 may be a novel marker for decreased ovarian function associated with the aging process. Although little is known about the functional aspects (role, hormone dependence, etc.) of pseudogene cyclin D2, the structural characteristics of pseudogene cyclin D2 compared to cyclin D2 have been studied. It has three internal stop codons (nucleotide positions 495, 956, and 1310), two frameshifts (positions 1188 and 1291), one insertion and deletion, and many missense mutations. To evaluate endometrial pseudogene cyclin D2 mRNA expression, we performed RT-PCR for pseudogene cyclin D2 in seven patients (aged 33 to 50). There was no endometrial pseudogene cyclin D2 mRNA expression in all seven patients regardless of their age (data was not shown), which suggests that pseudogene cyclin D2 mRNA expression may be an ovary-specific phenomenon.

Based on these preliminary results, the expression of pseudogene cyclin D2 mRNA in the human ovary may be a novel marker for decreased ovarian function associated with the aging process.

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